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Specification and Drawings, as originally filed, with Application for Patent Serial No:
2,245,903, on September 28, 1998, by **McGILL UNIVERSITY**, assignee of Andrew C.
Karaplis, David Goltzman, Mark L. Lipman and Janet E. Henderson, for "Use of Pex
in the Treatment of Metabolic Bone Diseases".

PRIORITY DOCUMENT

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ABSTRACT OF THE INVENTION

The present invention relates to a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition. The present invention also relates to a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.

USE OF PEX IN THE TREATMENT OF METABOLIC BONE DISEASESBACKGROUND OF THE INVENTION(a) Field of the Invention

5 The invention relates to the use of PEX in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

(b) Description of Prior Art

10 Mutations in the PEX gene are responsible for X-linked hypophosphatemic rickets (HYP). To gain insight into the role of PEX in normal physiology we have cloned the human full-length cDNA and studied its tissue expression, subcellular localization, and peptidase activity. We show that the cDNA encodes a 749
15 amino acid protein structurally related to a family of neutral endopeptidases that include neprilysin (NEP) as prototype. By Northern blot analysis, the size of the full-length PEX transcript is 6.5 kb. PEX expression, as determined by semi-quantitative PCR, is high in bone
20 and in tumor tissue associated with the paraneoplastic syndrome of renal phosphate wasting. PEX is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase from Triton X-114 extractions of transiently
25 transfected COS cells. Immunofluorescence studies in A293 cells expressing PEX tagged with a c-myc epitope show a predominant cell-surface location for the protein with its C-terminal domain in the extracellular compartment, substantiating the assumption that PEX,
30 like other members of the neutral endopeptidase family, is a type II integral membrane glycoprotein. Cell membranes from cultured COS cells transiently expressing PEX efficiently degrade exogenously added
35 PTH-derived peptides, demonstrating for the first time that recombinant PEX can function as an endopeptidase.

PEX peptidase activity may provide a convenient target for pharmacological intervention in states of altered phosphate homeostasis and in metabolic bone diseases.

X-linked hypophosphatemic rickets (HYP) is the most common inherited disorder of renal phosphate wasting characterized by severe hypophosphatemia, renal phosphate wasting, reduced serum concentrations of 1,25-dihydroxyvitamin D levels, and defective bone mineralization. Until recently, much of our understanding of HYP has been facilitated by the availability of two murine homologues, the *Hyp* and *Gy* mice, which exhibit many of the phenotypic features of HYP. Through positional cloning, however, a gene which spans the deleted region Xp22.1 in HYP patients, or is mutated in non-deletion patients with the disorder, was identified (designated *PEX*) and its partial cDNA sequence reported (The HYP Consortium (1995) *Nature Genetics* 11, 130-136). The predicted human *PEX* gene product, as well as its murine homologue (Du, L. et al. (1996) *Genomics* 36, 22-28), exhibit homology to a family of neutral endopeptidases involved in either activation or degradation of a number of peptide hormones. It has been postulated that *PEX* metabolizes a peptide hormone that modulates renal tubular phosphate handling. Such an activity could involve either the processing of a phosphate-reabsorbing hormone precursor to its active form or the inactivation of a circulating phosphaturic factor. These speculations notwithstanding, the physiologic function of the *PEX* gene product and the mechanisms that lead to the renal and skeletal abnormalities of HYP remain to be defined.

Oncogenous hypophosphatemic osteomalacia (OHO) is a rare acquired disorder of phosphate homeostasis with biochemical and physical abnormalities similar to HYP. This syndrome is associated with a variety of

histologically distinct, usually benign, mesenchymal tumors whose excision promptly reverses the metabolic abnormalities and results in cure of the bone disease. It is generally thought that a factor(s) produced by these tumors promotes phosphaturia and inhibits the renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. The nature of the phosphaturic substance remains unknown and is likely distinct from both parathyroid hormone (PTH) and calcitonin, two polypeptide hormones known to inhibit the renal tubular reabsorption of phosphorus. Because of the striking similarity in the clinical presentation of patients with OHO and HYP, it is postulated that the factor causing phosphaturia in OHO is the active form of the PEX substrate. The identification and characterization of the putative PEX substrate, referred to as phosphatonin, however, will require first a better understanding of PEX function.

To date, there is still a need to understand how local factors produced in the bone regulate bone formation and bone resorption. Derangement of these factors leads to metabolic bone diseases. Pharmacological manipulation of such factors may serve as a novel approach to the treatment of these disorders.

It would be highly desirable to be provided with a tool in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

30 SUMMARY OF THE INVENTION

One aim of the present invention is to provide a tool in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

Another aim of the present invention is to provide the use of *PEX* in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

Another aim of the present invention is to
5 provide a method of diagnostic of metabolic bone diseases, such as osteomalacia and osteoporosis.

Toward this objective, we have cloned a cDNA encoding the full-length human *PEX* protein, and determined the tissue distribution of *PEX* transcripts.
10 In addition, we have examined the subcellular localization of recombinant *PEX* protein and demonstrated its peptidase activity.

In accordance with the present invention there is provided a method for the diagnosis of metabolic
15 bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases
20 predisposition.

In accordance with the present invention there is provided a method for the treatment of metabolic bone diseases, which comprises administering to a patient a compound for the modulation of *PEX* enzymatic
25 activity.

In accordance with the present invention there is provided a method for the treatment of metabolic bone diseases, which comprises modulating PTH and PTHrP levels that regulate osteoblast activity in a patient
30 to modulate bone breakdown and bone formation.

In accordance with the present invention there is provided a transgenic non-human mammal to study the
~~role of *PEX* in bone development and homeostasis, whose~~
germ cells and somatic cells contain a *PEX* gene
35 construct for expression of *PEX* in osteoblast con-

sisting essentially of a recombinant PEX gene sequence under the control of a proximal promoter of a pro- α 1(I) collagen gene, the PEX gene construct being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

The non-human mammal is preferably a mouse and the proximal promoter is preferably murine pro- α 1(I) collagen gene, more preferably a 2.3 kb fragment thereof.

For the purpose of the present invention the following terms are defined below.

The expression "metabolic bone diseases" includes, without limitation, osteomalacia, osteoporosis, osteopetrosis and Paget's disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates PEX mRNA expression in OHO tumors;

Figs. 2A-2B illustrate human PEX cDNA cloned from OHO tumors;

Fig. 3 illustrates PEX expression in human tissues;

Fig. 4 illustrates a Northern blot analysis of PEX mRNA;

Fig. 5 illustrates *in vitro* translation of human PEX cRNA;

Figs. 6A-6B illustrate TRITON™ X-114 extraction and immunofluorescent localization of PEX;

Figs. 7A-7C illustrate HPLC analysis of the hydrolysis of [D-Ala²,Leu⁵]enkephalin;

Figs. 8A-8C illustrate the hydrolysis of PTH-derived peptides by PEX endopeptidase activity; and

Fig. 9 illustrates Schematic representation of phosphate handling in the proximal renal tubule in normal, OHO, and HYP states.

DETAILED DESCRIPTION OF THE INVENTION***PEX is a Cell Membrane-Associated Protein***

5 Previous studies have established that NEP, ECE-1 and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent TRITON™ X-114 and immunochemical localization to examine whether PEX is also a membrane-associated protein. For
10 identification of PEX, we generated a construct in which the carboxyl terminus sequences of PEX are modified by a human c-myc tag. The epitope tag was inserted immediately upstream of the potential prenylation motif so that any lipid modification of the PEX protein may proceed
15 uninterrupted.

 TRITON™ X-114 is a detergent that forms an aqueous solution at 4°C but separates into hydrophobic and aqueous phases when the temperature is raised to 30-37°C. This property has been used as an indicator of the
20 hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. TRITON™ X-114 extracts from COS-7 cells transiently expressing PEX tagged with the c-myc epitope
25 showed that PEX partitions nearly exclusively into the detergent phase. This finding indicates that PEX is a membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

30 To determine the subcellular localization of PEX, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, myc-tagged PEX immunostaining was detected
35 primarily on the cell surface, but in a number of cells

staining was also observed intracellularly, although no signal was observed in the nucleus. If permeabilization was omitted, staining was localized exclusively to the plasma membrane, while untransfected cells or cells
5 transfected with vector alone showed no immunofluorescent staining. Since the myc-tag was inserted in the carboxyl end of PEX, these findings further corroborate the sequence-based prediction that PEX is a Type II integral membrane protein with its large C-terminal hydrophilic
10 domain containing the active enzymatic site in the extracellular compartment.

Recombinant PEX protein has peptidase activity

The subcellular localization and sequence similarity between PEX and NEP strongly suggest that PEX
15 functions as a membrane-bound metallopeptidase. However, no peptidase activity has yet been ascribed to PEX. As shown, when [D-Ala², Leu⁵] enkephalin, used to assay for NEP activity, was incubated with cell membrane preparations from vector-transfected COS cells or COS
20 cells expressing equivalent amounts of recombinant human NEP or PEX proteins, as determined by Western blot analysis, production of Tyr-D-Ala-Gly from the substrate was evident only in NEP-expressing membrane preparations. While the PEX sequence preserves two of the residues
25 critical for catalytic activity of NEP (equivalent to E⁶⁴⁶ and H⁷¹¹), it lacks a residue equivalent to R¹⁰² shown to be crucial for the dipeptidylcarboxypeptidase activity of NEP. Therefore, unlike NEP, PEX has no dipeptidylcarboxypeptidase activity.

30 To test for peptidase activity of recombinant PEX, cell membrane preparations from vector-transfected COS cells or COS cells expressing recombinant PEX protein were incubated with human parathyroid hormone PTH (1-34) and PTH (1-38). As shown, PEX activity was able to
35 degrade both peptides in a very characteristic pattern.

Therefore, *PEX* functions as an endopeptidase, and more specifically we have shown for the first time that it degrades PTH. PTH is the first and only known substrate of *PEX*.

5 These observations make two important points:

PEX is a membrane bound protein with its active enzymatic site in the extracellular compartment. The cells with the highest level of *PEX* expression are the osteoblasts (bone forming cells). These cells are also
10 the site of action of circulating PTH at the level of the bone. PTH stimulates these cells to produce factors (nature unknown) which in turn stimulate other bone cells, specifically the osteoclasts, to break down bone. Since *PEX* likely inactivates PTH in contact with
15 osteoblasts, it would result in decreased stimulation of osteoclasts and therefore less bone breakdown.

Alternatively, osteoblasts produce parathyroid hormone-related peptide, PTHrP, which is important in the development of normal bone density. PTHrP shares many of
20 the structural features of PTH and may therefore also serve as substrate for *PEX*. Our previous studies using PTHrP heterozygous-null mice generated by gene targeting have shown that decreased levels of PTHrP in the skeletal microenvironment lead to a premature form of
25 osteoporosis. *PEX* in osteoblasts may therefore modulate local PTHrP levels and thus bone formation. Inhibition of *PEX* enzymatic activity may allow higher local concentrations of PTHrP and therefore better bone formation.

30 By examining PTH breakdown fragments, we can now design peptide and non-peptide activators and inhibitors of *PEX* enzymatic activity.

By modulating PTH and PTHrP levels that regulate osteoblast activity, *PEX* may play a critical role in the
35 pathogenesis of osteomalacia and osteoporosis. By

pharmacological modulation of PEX activity, it will be possible to modulate bone breakdown and bone formation. This would be a totally novel approach to the treatment of these metabolic bone diseases.

5

Experimental procedure

Tumor Tissues

Patient I was a 55 year-old woman who presented with a two year history of progressively increasing bone pain and difficulty in walking. X-rays of the lumbosacral spine showed diffuse osteopenia. Biochemical investigation showed the serum calcium level to be normal while serum phosphorus was low (0.41 to 0.57 mmol/L; normal, 0.8-1.6 mmol/L). Alkaline phosphatase was 232 U/L (normal, 30-105 U/L) and tubular reabsorption of phosphate while the patient was hypophosphatemic was decreased to 63% (normal, >80%). A search for a tumor was negative and the patient was treated with 1,25-dihydroxyvitaminD3 and oral phosphate. Five years later a right hand mass was discovered and was surgically removed. On histopathological examination, it was a fibrous hemangioma. Postoperatively, the patient noted increasing strength in her lower extremities and marked decrease in her pain. The serum phosphorus normalized (0.96 mmol/L) and the tubular reabsorption of phosphate improved but did not completely normalize (71-76%). No recurrence of the tumor has been found ten years later.

Patient II was a 21 year old man with classic features of OHO. Resection of a benign extraskeletal chondroma from the plantar surface of the foot resulted in complete reversal of the biochemical and clinical abnormalities associated with the syndrome.

Tumor tissue obtained from these two patients at surgery was frozen immediately in liquid nitrogen and stored at -70°C .

PEX Expression in OHO-Associated Tumors

5 RNA was extracted from tumor tissue using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA) and reverse transcribed using oligodT primer and Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of $30\ \mu\text{l}$. The
10 resulting cDNA was then amplified using human PEX-specific oligonucleotide primers PEX-1 (5'-GGAGGAATTGGTTGAGGGCG -3') and PEX-2 (5'-GTAGACCACCAAGGATCCAG -3'), designed from the published cDNA sequence (1298 and 1807 are the nucleotide
15 positions of the 5' end of the sense and antisense primers, respectively) (The HYP Consortium (1995) *Nature Genetics* 11, 130-136). Following amplification (35 cycles), an aliquot of the PCR reaction was fractionated on a 1% agarose gel and visualized
20 following staining with ethidium bromide.

Cloning of Full-Length PEX cDNA

Cloning of the 5' end of PEX cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumor II and mRNA was prepared. 1.5 μg
25 of mRNA was reverse transcribed into cDNA using 100 ng of a PEX-specific antisense oligomer (PEX-2) and 200 units of Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of $30\ \mu\text{l}$. The resulting cDNA was size fractionated on a 1% agarose
30 gel and fragments corresponding to $>600\ \text{bp}$ were purified and resuspended in H_2O . The 3' end of the first strand cDNA was homopolymer tailed with dGTP using $1\ \mu\text{l}$ of Terminal deoxynucleotidyl transferase
(TdT) at 37°C for 30 minutes in a volume of $50\ \mu\text{l}$.
35 Following heat inactivation of the enzyme, the RNA

template was removed by incubation with RNase H and the tailed cDNA was purified by phenol-chloroform extraction followed by ammonium acetate precipitation. The purified tailed cDNA was resuspended in H₂O and an aliquot was used for anchored PCR analysis along with 200 ng of an internal PEX specific antisense primer (PEX-3, 5'-CGTGCCCGAACTAGGGTGCCACC-3'; nucleotide 98 of the published human cDNA sequence is the 5' end of the primer) and 200 ng of oligodC as the sense primer. Forty cycles of PCR were performed using 0.5 μ l of Taq polymerase (Promega Biotec, Madison, WI) in a reaction volume of 50 μ l. Cycling parameters were: 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C and 2 minutes of extension at 72°C. The PCR products were fractionated on a 1% agarose gel and a band of 700 bp was isolated, purified, and ligated into pPCRII vector (Invitrogen). Following transformation into INV F' bacteria, clones containing the appropriate size insert were sequenced.

To clone the 3' end of PEX cDNA, an aliquot of an amplified unidirectional cDNA library in pCDNA3 vector (Invitrogen) generated from mRNA obtained from tumor I was grown overnight in LB medium and plasmid DNA extracted. DNA (0.5 μ g) was subjected to PCR using a PEX-specific sense oligomer (PEX-1) and an antisense oligomer corresponding to the SP6 RNA polymerase binding site sequences present in the pCDNA3 vector. Thirty-five cycles of amplification were performed in a 50 μ l reaction volume with each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. Amplified products were fractionated on a 1% agarose gel and a 1.2 kb fragment corresponding to the 3' end of PEX cDNA was subcloned and sequenced.

For expression studies, an EcoRV (in the polylinker of pPCR11) /AccI (in the PEX sequence) fragment containing the 5' end of PEX cDNA was ligated into the pPCR11 vector containing the 3' end of PEX cDNA following digestion with AccI and EcoRV. The resulting plasmid was restricted with KpnI and NotI excising the full length PEX cDNA that was then inserted into pCDNA3 vector digested at the KpnI/NotI sites in the polylinker region, resulting in plasmid pPEX. The full-length PEX cDNA was sequenced using an Applied Biosystems 373A automated sequencer.

Tissue Expression of PEX mRNA

PEX expression was examined in normal human tissues and in the Saos-2 human osteoblastic osteosarcoma cell line, by RT-PCR using oligonucleotides PEX-4 (5'-CTGGAT-CCTTGGTGGTCTAC-3') and PEX-5 (5'-CACTGTGCAACTGTCTCAG-3') as sense and antisense primers (2398 and 2895 are the nucleotide positions of the 5' end of these primers designed from the full-length human PEX cDNA). Semiquantitative PCR analysis for PEX expression in human tissues was performed as previously described, following normalization for GAPDH message in all samples containing PEX transcripts.

Northern-blot Analysis

Total RNA was obtained from Tumor I and human Saos-2 osteosarcoma cells using the RNeasy Total RNA kit (Qiagen) and oligo(dT)-purified poly(A)⁺ RNA was isolated from Saos-2 total RNA using standard procedures. Twenty micrograms of Tumor I total RNA and 20 µg of Saos-2 poly(A)⁺ RNA were fractionated on 1% denaturing agarose gel, and transferred to nylon membrane (Hybond N⁺, Amersham). Hybridization was performed with 32p-labeled full-length human PEX cDNA (3.1 kb) in 7 mM Tris-HCl, 50% formamide, 10% dextran

sulfate, 4 X SSC, 2 x Denhardt's solution and heat-denatured salmon sperm DNA (100 µg/ml). The blot was washed in 0.1 X SSC, 0.1% SDS for 20 min at 50°C, and subjected to autoradiography for 4 days.

5 *In Vitro Transcription, Translation, and Analysis of Products*

Plasmid pPEX was linearized with NotI and sense RNA strand was transcribed using T7 RNA polymerase. Translation reactions in rabbit reticulocyte lysate were performed in the presence of [³H]leucine according to the manufacturer's recommendations (Promega) with or without canine pancreas microsomal membranes. Products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 8%). Autoradiography was performed after treating the gel with EN³HANCE (Dupont NEN), as previously described.

Generation of myc-tagged PEX, Transfection in COS-7 Cells, and Triton X-114 Extraction

Plasmid pPEX-myc was generated by PCR amplification of PEX cDNA using oligonucleotide PEXMyc1 as the sense primer (5'-TTGGATGTCAACGCCTCG -3', 519 is the nucleotide position of the 5' end of this primer designed from the cloned human PEX cDNA) and PEXMyc2 as the antisense (5'-CTACCACAATCTACAGTTGTTTCAGGTC-CTCTTCGCTAATCAGCTTTTGTTCATAGAGTCCATGCCTCTG-3') primer. The latter encodes the human c-myc tag sequences (underlined) and PEX sequences corresponding to the carboxyl terminal of the mature protein (742RGMDSMEOKLISEEDLNNCRLW*). Following PCR, the amplified fragment was ligated to the pPCR II vector, excised by digestion with KpnI/NotI and inserted into the corresponding sites in the polylinker region of pCDNA3. ~~The in-frame fusion protein was verified by DNA sequencing.~~

35 COS-7 cells maintained in Dulbecco's modified Eagle's medium (DMEM, 4,500 mg/L glucose with L-

glutamine; JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (FCS; GIBCO) and antibiotics (pen/strep) were plated at a density of 3×10^5 cells/well in 6-well cluster plates 24 h prior to transfection. Cells were washed with twice with PBS and incubated with 2 μ g of pPEX-myc plasmid DNA in 1 ml of DMEM containing 0.1% BSA, and DEAE-dextran (Pharmacia LKB) for 3.5 h at 37°C. Following incubation, the transfection medium was aspirated, the cells were shocked with 10% DMSO in PBS for 2 min, and then cultured in DMEM with 10% calf serum at 37°C for 48 h. Triton X-114 extraction were performed on cultured cells expressing myc-tagged PEX as described. The samples were then analyzed by immunoblotting using the 9E10 anti-myc monoclonal antibody.

Stable Transfection of A293 Cells and Immunofluorescence

A293 cells maintained in DMEM with 10% FCS were transfected with the pPEX-myc plasmid by electroporation and selection initiated using G418 (600 mg/ml for 14 days and then decreased to 400 mg/ml). Populations of stably transfected cells were recovered at the end of the selection period. For myc-tagged PEX indirect immunofluorescence, stably transfected cells plated on gelatin-coated coverslips were washed twice with PBS, fixed in 4% paraformaldehyde and in some experiments permeabilized with 0.5% Triton X-100. Cells were blocked with 10% FCS in DMEM for 30 min, washed and incubated for 1 hr at 37°C with the 9E10 anti-myc monoclonal antibody (1:500 dilution). Cells were subsequently washed and incubated in turn with fluorescein-conjugated sheep anti-mouse secondary antibody (1:250 dilution). Coverslips were rinsed extensively with PBS, mounted in medium (glycerol:Tris; 1:1) containing 2.5% 1,4-diazabicyclo-(2,2,2) octane

(Sigma) and examined with fluorescent microscopy using appropriate filters.

Assay for membrane-bound endopeptidase activity

COS-7 cells transiently transfected with pCDNA3
5 vector alone, with vector containing human *NEP* cDNA
(generous gift of P. Crine, Université de Montréal), or
with pPEX plasmid, were washed and scraped in PBS.
Following brief centrifugation, the cell pellets were
resuspended in 50 mM Tris-HCl, pH 7.4 and disrupted by
10 sonication. Homogenates were fractionated by sequential
centrifugation at 1,000 x g for 10 min and then at
100,000 x g for 60 min. The final precipitate was
washed with 50 mM Tris-HCl, pH 7.4, resuspended in the
same buffer, and assayed for endopeptidase activity.
15 The protein concentration in membrane fractions was
determined by the method of Bradford with bovine serum
albumin as standard.

[D-Ala²,Leu⁵] enkephalin (500 µM) was incubated
with COS cell membrane preparations (~60 µg of protein)
20 in 100 mM Tris-HCl, pH 7.0, at 37°C for 30 min (final
volume 30 µl). The reaction was terminated by the
addition of 100 µl 0.1% TFA (v/v). Production of Tyr-D-
Ala-Gly was monitored using reversed-phase HPLC
(Bondpak C-18 reverse phase column, Waters) with a U.V.
25 detector set at 214 nm. A linear solvent gradient of 0%
B to 40% B in 60 min was used with a flow rate of 1.5
ml/min (mobile phase A=0.1% TFA (v/v); mobile phase
B=80% acetonitrile/0.1% TFA). Tyr-D-Ala-Gly was
identified by co-chromatography with marker synthetic
30 peptide. For assessing PEX endopeptidase activity, 10
µg of PTH [1-38] and PTH [1-34] peptides (Peninsula
Laboratories; Belmont, CA) were added to the membrane
preparations. For HPLC analysis of hydrolysis products,
a linear solvent gradient of 0% to 50% solution B was
35 used at a rate of 1.5 ml/min. MALDI-TOF mass

spectrometry was performed on specific peptide fragments.

RESULTS

Cloning of Human PEX cDNA

5 At the initiation of these studies, PEX
expression had been reported in minute amounts only in
leukocytes and fetal brain. We postulated that in
states of hypophosphatemia PEX expression may be
increased and therefore opted to use the OHO tumor as a
10 tissue source that may express considerably more PEX.
Tissues obtained from two tumors associated with OHO
were used to obtain total RNA and analysis for PEX mRNA
expression was assessed by RT-PCR. As shown in Fig.1,
PEX transcripts were readily amplified from both tumor
15 samples demonstrating the expected 509 bp fragment
predicted from the published partial human PEX sequence
(The HYP Consortium (1995) Nature Genetics 11, 130-
136). Total RNA extracted from two tumors associated
with OHO was reverse transcribed and amplified by PCR
20 (35 cycles) using human PEX-specific primers, PEX-1 and
PEX-2, designed from the published human sequence. The
expected 509 bp amplified fragment was obtained from
both tumor samples. Control, no cDNA added to the
amplification reaction, i.e. negative control; Marker,
25 174 DNA digested with HaeIII restriction endonuclease.

 The cloning of the 3' end of PEX transcript was
performed by rapid amplification of the 3' end of the
cDNA (3' RACE), while the 5' of the cDNA was amplified
by anchored PCR, as described in Experimental
30 Procedures. Fig. 2A shows the nucleotide and predicted
amino acid sequence of the full-length human PEX cDNA
cloned from tumor tissues. Nucleotide and deduced amino
acid sequence of tumor-derived human PEX cDNA
(Fig. 2A). The numbering begins at the 5' end
35 nucleotide as determined by anchored PCR. Amino acids

are given below each codon using the single letter code. The putative start codon is indicated as /1 along with the deduced amino acid translation. Two stop codons preceding the predicted initiation ATG are in bold type. Asterisk (*) indicates an in-frame stop codon, while a large asterisk (*) denotes the putative prenylation site. A potential polyadenylation signal in the 3' untranslated region is underlined. Nine potential N-glycosylation sites are boxed. The sequence has been assigned GenBank accession No. (U82970).

The composite cDNA reveals a single open reading frame encoding a protein of 749 amino acids which displays homology (34.2% identity, 70% similarity) to human neprilysin (NEP; EC 3.4.24.11), and other members of the membrane-bound metalloendopeptidase family encompassing endothelin-converting enzyme-1 (ECE-1; 66% similarity) and the Kell antigen (60% similarity), suggesting that PEX is a novel member of this family of neutral endopeptidases, as previously suggested (The HYP Consortium (1995) *Nature Genetics* 11, 130-136). Like the other members, PEX is a likely a glycoprotein with eight potential N-glycosylation sites and 10 cysteine residues that may be important for the proper folding and hence native conformation of the protein.

The ATG codon at position 604 was assigned as the initiator methionine since it is preceded by two in-frame TGA termination codons 36 and 63 basepairs upstream and conforms favorably to the Kozak consensus for vertebrate initiation of translation. The cloned cDNA identifies the first 3 and the last 108 amino acids of the predicted PEX gene product in addition to the published partial sequence. These additional amino acids comprise residues such as E⁶⁴² and H⁷¹⁰ that are shared by NEP, and may be critical for the formation of

the active site of the protein and hence its enzymatic activity. Three amino acid residues predicted from our cDNA clone differ from the published partial human PEX sequence, D363A (GAC to GCC), R403W (AGG to TGG), and A641G (GCG to GGA). To confirm that these alterations did not arise because of PCR errors, PEX sequences were amplified from Saos-2 human osteosarcoma cells (see below) and sequenced. In addition, the same alterations were subsequently described in the murine PEX cDNA, suggesting possible cloning artifacts in the published partial human PEX sequence. Our cloned sequences also encompass 603 nucleotides of the 5' untranslated region, and 276 nucleotides of the 3' untranslated region, including the canonical polyadenylation signal AATAAA, 19 nt upstream of the poly(A) tract. The human and the published mouse PEX cDNA sequences share extensive homology within the protein coding region (96% identity) as well as in the 5' and 3' non coding regions.

TMpred analysis of the human PEX sequence predicts that the protein has no apparent N-terminal signal sequence but has a single membrane-spanning helical domain comprising amino acid residues 21-39 (Fig. 2C). TMpred analysis of the PEX sequence showing a single membrane-spanning domain encompassing amino acid residues 21-39 (arrowhead). Numbers on the horizontal axis refer to the amino acid sequence. Amino acid homology between PEX and human NEP cDNA (Fig. 2B). Sequence comparison was performed using the LALIGN program.

This predicts its transmembrane topology to be that of a type II integral membrane protein with a 20-residue N-terminal cytoplasmic tail and a C-terminal of 700 amino acid residues containing the catalytic domain in the extracellular compartment. Unexpectantly, a CXXX

box motif comprising amino acid residues 746CRLW was also identified at the carboxyl terminus of PEX. This motif may serve as a site for prenylation, a post-translational lipid modification involved in a number of processes including facilitating membrane attachment, targeting of proteins to specific subcellular membrane compartments, promoting protein-protein interactions and regulating protein function.

Tissue Expression of PEX mRNA

We next examined PEX expression in a number of fetal and adult tissues and compared the level of expression to OHO tumor RNA using semi-quantitative RT-PCR (Fig. 3). Quantitative RT-PCR amplification of the PEX transcripts from total RNA prepared from human tissues and OHO-associated tumor. Relative expression levels for the PEX transcript were measured by quantifying PEX product in reversed-transcribed RNA samples that have been previously normalized for GAPDH levels. The specific primers used were as follows: for PEX, the forward primer was PEX-4 and the reverse primer PEX-5; for GAPDH, the primers were as previously described. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Control, negative control; Marker, 174 DNA digested with HaeIII restriction endonuclease. Below, shown are the relative levels of PEX transcripts in various human tissues compared to those in the tumor.

PEX transcripts were expressed in human fetal calvarium and to a lesser degree in fetal kidney and skeletal muscle while no expression was apparent in fetal liver. PEX expression was also observed in the human osteoblastic osteosarcoma cell line, Saos-2. In adult tissues, PEX mRNA was identified in kidney, but not in liver, or endomyocardium. Recent studies have also reported PEX expression in human fetal bone,

skeletal muscle, and liver as well as fetal and adult ovary and lung (Beck, L. et al. (1997) *J. Clin. Invest.* 99, 1200-1209; Grief, M. et al. (1997) *Biochem. Biophys. Res. Commun.* 231, 635-639). Analysis following
5 normalization for GAPDH message in all tissues containing PEX transcript disclosed that bone PEX expression is 2-10 fold higher than in other normal tissues examined. In comparison, OHO tumor PEX
10 expression was twice the levels observed in fetal calvarium, consistent with its relative "overabundance" in these tissues.

Northern Blot Analysis

To determine the size of the full-length PEX transcript, we isolated total RNA from tumor I
15 (quantity of available tissue was insufficient for poly(A)⁺ RNA extraction) and poly(A)⁺ RNA from human Saos-2 osteosarcoma cells. This cell line was used since it is readily available and successful
20 amplification of PEX sequences has been performed by RT-PCR (see above). Aliquots (20 µg of each) were examined by Northern-blot analysis using the cloned human PEX cDNA as probe. A single transcript of
25 approximately 6.5 kb was readily detected only in the Saos-2-derived poly(A)⁺ sample and contrasts with the predicted size of the cloned sequence of 3.1 kb
(Fig. 4). Approximately 20 µg of poly(A)⁺ RNA prepared from Saos-2 cells and 20 µg of total RNA prepared from
30 tumor I tissue were resolved on 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. Following hybridization with radiolabeled PEX cDNA, the blot was washed and the signal detected by
autoradiography. A transcript of ~6.5 kb was observed
only in the lane containing Saos-2 poly(A)⁺ RNA. There
is suggestion of an additional band corresponding to a
35 transcript of ~3.8 kb. Arrows indicate the position of

the 28S (approx. 4.8 kb) and 18S (approx. 1.8 kb) ribosomal RNA.

This finding would therefore predict a ~4 kb 5' untranslated region for *PEX* cDNA, consistent with published data from Northern blot analysis of *PEX* expression in mouse calvaria (Du, L. et al. (1996) *Genomics* 36, 22-28). A less well defined band was also detected in the Saos-2 sample corresponding to a potential transcript of ~3.8 kb, although the nature of this transcript remains unclear. Northern analysis of total RNA samples from tumor I and Saos-2 cells (results not shown) did not reveal any signal for *PEX*, consistent with the relatively low expression levels of the *PEX* transcript, previously described (The HYP Consortium (1995) *Nature Genetics* 11, 130-136; Beck, L. et al. (1997) *J. Clin. Invest.* 99, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* 231, 635-639). This finding contrasts sharply with *PEX* expression levels demonstrated in murine calvaria and cultured osteoblasts (Du, L. et al. (1996) *Genomics* 36, 22-28) and may reflect tissue and species differences.

In vitro translation of PEX cRNA

In vitro translation studies using full-length human *PEX* cRNA were performed in the rabbit reticulocyte lysate cell-free system. In the absence of microsomal membranes, *PEX* cRNA was translated into an ~86 kD protein, as predicted from the cloned cDNA sequence (Fig. 5). Plasmid pPEX was linearized and sense RNA strand transcribed using T7 RNA polymerase. Translation of *PEX* cRNA was performed using rabbit reticulocyte lysate in the absence (minus) and presence (plus) of canine pancreas rough microsomes. Products were electrophoresed in a SDS-polyacrylamide gel (10%) and visualized by autoradiography. Arrowhead in lane 2 indicates full-length human *PEX* protein. The addition

of microsomal membranes results in the appearance of higher molecular weight forms that likely represent glycosylated products.

Following addition of canine microsomal membranes to the translation mixture, products of higher molecular weight (~100 kD) became apparent, consistent with N-glycosylation of PEX at the eight potential glycosylation sites deduced from the predicted sequence.

10 **PEX is a Cell Membrane-Associated Protein**

Previous studies have established that NEP, ECE-1 and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent Triton X-114 and immunofluorescent localization to examine whether PEX is also a membrane-associated protein. For identification of PEX, we generated a construct in which the carboxyl terminus sequences of PEX are modified by a human c-myc tag. The epitope tag was inserted immediately upstream of the putative prenylation motif so that any potential lipid modification of the PEX protein may proceed uninterrupted.

Triton X-114 is a detergent that forms an aqueous solution at 4°C but separates into hydrophobic and aqueous phases when the temperature is raised to 30-37°C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. Triton X-114 extracts from COS-7 cells transiently expressing PEX tagged with the c-myc epitope showed that PEX partitions nearly exclusively into the detergent phase (Fig. 6A). Extraction and partitioning of PEX expressed in COS-7 cells with Triton X-114 (Fig. 6A). Plasmid pPEX-myc was

transiently transfected in COS-7 cells and 48 h later cells were extracted with Triton X-114. Whole cell extracts, as well as detergent and aqueous phases, were analyzed by SDS-PAGE and immunoblotted with an anti-myc monoclonal antibody. Right margin indicates $M_r \times 10^{-3}$.

This finding indicates that *PEX* is a membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

To determine the subcellular localization of *PEX*, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, myc-tagged *PEX* immunostaining was detected primarily on the cell surface, but in a number of cells staining was also observed intracellularly, although no signal was observed in the nucleus (Fig. 6B). If permeabilization was omitted, staining was localized exclusively to the plasma membrane (Fig. 6C), while untransfected cells or cells transfected with vector alone showed no immunofluorescent staining. Localization of *PEX* using indirect immunofluorescence in stably transfected A293 cells with (Fig. 6B) and without (Fig. 6C) permeabilization with Triton X-100, respectively. Staining was carried out using the 9E10 anti-myc monoclonal antibody, followed by fluorescein-labeled secondary (sheep anti-mouse) antibody. Arrowheads indicate intracellular (B) and plasma membrane staining (C).

Since the myc-tag was inserted in the carboxyl end of *PEX*, these findings further corroborate the sequence-based prediction that *PEX* is a type II integral membrane protein with its large C-terminal hydrophilic domain in the extracellular compartment.

Recombinant PEX protein has endopeptidase activity

The subcellular localization and sequence similarity between PEX and NEP strongly suggest that PEX functions as a membrane-bound metallopeptidase. However, no peptidase activity has been ascribed to PEX. As shown in Fig. 7A, when [D-Ala², Leu⁵]enkephalin, used to assay for NEP activity, was incubated with cell membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human NEP or PEX proteins, as determined by Western blot analysis, production of Tyr-D-Ala-Gly from the substrate was evident only in NEP-expressing membrane preparations. Cell membrane preparations from vector transfected COS-7 cells (Fig. 7A) or from cells transiently expressing human NEP (Fig. 7B) or, human PEX cDNAs (Fig. 7C) were incubated in the presence of [D-Ala²,Leu⁵]enkephalin (500 μ M) and hydrolysis products were resolved by HPLC as described in the *Experimental Procedures* section. Tyr-D-Ala-Gly was identified by chromatography of synthetic marker peptide.

While the PEX sequence preserves two of the residues critical for catalytic activity of NEP (equivalent to E646 and H711), it lacks a residue equivalent to R102 shown to be crucial for the dipeptidylcarboxypeptidase activity of NEP. Therefore, unlike NEP, PEX has no dipeptidylcarboxypeptidase activity, but likely functions as an endopeptidase.

To examine recombinant human PEX for endopeptidase activity, cell membrane preparations from COS cells transiently expressing the protein were incubated with human PTH [1-38] or PTH [1-34] and the cleavage products were analyzed by reverse-phase high pressure liquid chromatography (HPLC), as shown in Fig. 8. Human PTH [1-38] was incubated with cell

membrane preparations from vector transfected COS-7 cells (Fig. 8A) or from cells transiently expressing human *PEX* and hydrolysis products were resolved by HPLC (Fig. 8B). Chromatographic profile of products arising from the hydrolysis of PTH [1-34] when incubated with cell membranes from COS-7 cells transiently expressing *PEX* (Fig. 8C). The novel product with a molecular weight of 630 likely corresponds to the terminal pentapeptide DVHNF of human PTH [1-34].

A parallel preparation from vector transfected COS cells did not appreciably cleave PTH [1-38]. However, in the presence of *PEX*, both PTH peptides were hydrolyzed in a highly reproducible pattern resulting in the formation of several peaks that absorb at 214 nm. Mass spectrometry of the peptide materials recovered from two product peaks gave m/z values of 861 and 630, respectively. While the former product was present in hydrolysates from both PTH [1-38] and PTH [1-34], the latter product was identified only in the PTH [1-34] hydrolysate and likely corresponds to the carboxyl terminal pentapeptide DVHNF of human PTH [1-34]. These findings provide the first direct evidence that recombinant *PEX* possesses endopeptidase activity and suggest that its substrate specificity may not be restricted to the putative phosphatonin but may include other circulating hormones or perhaps bone-derived autocrine/paracrine regulatory factors that regulate renal phosphate handling.

DISCUSSION

To gain insight into the role of *PEX* in normal physiology we have cloned the human full-length cDNA and studied its expression, subcellular localization, and peptidase activity. The cloned human *PEX* cDNA encodes a protein whose deduced amino acid sequence is identical to the published partial (The HYP Consortium

(1995) *Nature Genetics* 11, 130-136) and to the full-length sequences reported more recently (Beck, L. et al. (1997) *J. Clin. Invest.* 99, 1200-1209; Grief, M. et al. (1997) *Biochem. Biophys. Res. Commun.* 231, 635-639; Guo, R. and Quarles, L. D. (1997) *J. Bone Miner. Res.* 12, 1009-1017). Its deduced topology is that of a type II integral membrane glycoprotein and in the present study we have provided experimental evidence to support this prediction. We have shown that PEX is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase following extraction with Triton X-114, consistent with the prediction from sequence analysis that it is an integral membrane glycoprotein. Nevertheless, the observed hydrophobic nature of PEX, need not be attributed solely to it being an integral membrane protein. Lipophilic modification is known to cause cell membrane association, presumably through hydrophobic interaction of the modifying group with the lipid bilayer. Signaled by the C-terminal tetrapeptide CRLW motif, post-translational attachment of isoprenoids via a thioether linkage to the cysteine residue would be sufficient to promote effective membrane association. Further studies will be necessary to determine if such lipid modification of PEX does indeed take place. Of interest, however, is the observation that a nonsense mutation within this motif (R747Stop) has been reported to co-segregate with HYP and is likely to be associated with an inactive PEX gene product. Finally, the localization of PEX expressed in A293 cells is also consistent with the protein being membrane-associated and corroborates the sequence-based prediction that PEX is a type II integral membrane protein with its large C-terminal hydrophilic domain in the extracellular compartment.

While protein expression was detected mostly on the cell surface, in some cells the signal was also localized intracellularly. This localization of the expressed protein would indicate that a portion of *PEX* activity is located in a membrane-bound compartment, possibly the Golgi membranes. The Golgi localization described for ECE-1 activity in cultured endothelial cells is proposed to promote the efficient conversion of big endothelin-1 because of the co-localization and concentration of enzyme and substrate through the constitutive secretory pathway. It is possible then, that in parallel fashion, the *PEX* enzyme mediates both intracellular and cell-surface conversions of its putative substrate.

The finding that wild-type *PEX* transcripts are expressed in relative overabundance in OHO tumors poses a question in trying to understand the pathophysiology of these disorders. That is, how do we reconcile the apparently disparate observations that overexpression of *PEX* in OHO and loss of function in HYP patients, both lead to similar derangement in phosphate homeostasis? One of the physiological functions of *PEX* may well be the inactivation of a factor that normally promotes renal phosphate excretion (Fig. 9). The diagrams indicate events proposed to occur at the level of the proximal renal tubule. A putative circulating phosphaturic hormone (PHA) interacts with its renal receptor (PR) and inhibits phosphate reabsorption across the renal brush border membrane (-|) by decreasing NaPi activity. Downward arrows indicate the degree of phosphate excretion. *PEX* expressed predominantly in extrarenal tissues modulates the levels of circulating PHa by converting it to its inactive form (PHi).

In patients with OHO, the hyperphosphaturia that characterizes the syndrome would be the consequence of unregulated and excessive elaboration of the phosphaturic factor by the tumor. The modestly elevated PEX levels that we have documented in these tumors may arise either in response to the severe hypophosphatemia or to the abnormally high levels of the active phosphaturic factor. Yet, the increased PEX expression may not be sufficient to accommodate the increased substrate load, resulting in abnormally high circulating levels of the active phosphaturic hormone. The inactivation of PEX observed in HYP patients would similarly cause decreased turnover of this humoral phosphaturic factor and thereby lead to renal phosphate wasting.

This model is also consistent with the observation that the Hyp phenotype is neither corrected nor transferred following cross transplantation of kidneys in normal and Hyp mice. Thus, when Hyp mice are engrafted with a normal kidney, phosphaturia ensues since circulating levels of the phosphaturic agent are excessive. On the other hand, engraftment of mutant kidneys in normal mice will not affect renal tubular phosphate handling of the recipients since circulating levels of the phosphaturic substance will be normally regulated by the enzymatic activity of extrarenal wild-type PEX. Indeed, analysis of the tissue distribution of PEX mRNA by RT-PCR has confirmed its expression in extrarenal tissues and particularly bone. Our present findings and those of others (Du, L. et al. (1996) *Genomics* 36, 22-28; Beck, L. et al. (1997) *J. Clin. Invest.* 99, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* 231, 635-639; Guo, R. and Quarles, L. D. (1997) *J. Bone Miner. Res.* 12, 1009-1017) showing high levels of PEX expression in cells of

the osteoblast lineage would be consistent with the intrinsic osteoblast defect postulated to exist in HYP patients and in *Hyp* mice.

Finally, although the deduced structure of *PEX* clearly suggests that it is a metalloprotease, no peptidase activity had been ascribed to the protein. The preservation of the catalytic glutamate and histidine residues (equivalent to E⁶⁴⁶ and H⁷¹¹ of NEP; Fig. 2B) would argue for such an activity. In addition, the wide range of *PEX* mutations in HYP patients that align with regions required for protease activity in NEP suggests that *PEX* also functions as a protease. Here, for the first time, we provide experimental evidence that recombinant *PEX* indeed functions as an endopeptidase. Unlike NEP, however, the protein does not possess dipeptidylcarboxypeptidase activity since it lacks a residue equivalent to R¹⁰² of NEP. Our unexpected observation that *PEX* effectively degrades PTH raises the question of whether circulating PTH is the putative phosphatonin. Although extracts from some OHO tumors have been reported to stimulate renal adenylate cyclase and this activity was inhibited by PTH antagonists, most studies have excluded PTH and PTH-related peptide (PTHrP) activity in OHO-associated tumors. Moreover, calcium homeostasis is generally preserved in patients with HYP. It is more likely, therefore, that the enzyme is rather promiscuous in its substrate specificity. *PEX* may indeed modulate PTH bioavailability and bioactivity, particularly at the level of the osteoblast, as well as the hormonal and paracrine/autocrine effects of factors produced by osteoblasts involved in regulating phosphate reabsorption and osteoblast maturation and mineralization. Although additional work will be required to clarify many of these issues, the

availability of full-length human PEX cDNA now provides
us with the opportunity to study the biology of PEX,
identify its substrate(s), elucidate its role in
pathological states characterized by dysregulated
5 phosphate homeostasis, and determine its suitability as
target for therapeutic intervention in the treatment of
metabolic bone diseases.

While the invention has been described in con-
nection with specific embodiments thereof, it will be
10 understood that it is capable of further modifications
and this application is intended to cover any varia-
tions, uses, or adaptations of the invention following,
in general, the principles of the invention and
including such departures from the present disclosure
15 as come within known or customary practice within the
art to which the invention pertains and as may be
applied to the essential features hereinbefore set
forth, and as follows in the scope of the appended
claims.

WHAT IS CLAIMED IS:

1. A method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.
 2. The method of claim 1, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
 3. A method for the treatment of metabolic bone diseases, which comprises administering to a patient a compound for the modulation of PEX enzymatic activity.
 4. The method of claim 3, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
 5. A method for the treatment of metabolic bone diseases, which comprises modulating PTH and PTHrP levels that regulate osteoblast activity in a patient to modulate bone breakdown and bone formation.
 6. The method of claim 5, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
 7. A transgenic non-human mammal to study the role of PEX in bone development and homeostasis, whose germ cells and somatic cells contain a PEX gene construct for expression of PEX in osteoblast consisting essentially of a recombinant PEX gene sequence under the control of a proximal promoter of a pro- α 1(I) collagen gene, the PEX gene construct being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.
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8. The non-human mammal of claim 7, which is a mouse and the proximal promoter is murine pro- α 1(I) collagen gene.

9. The non-human mammal of claim 8, wherein said murine pro- α 1(I) collagen gene is a 2.3 kb fragment thereof.

Control
Tumor I
Tumor II
Marker



PEX



GAPDH

Fig. 1

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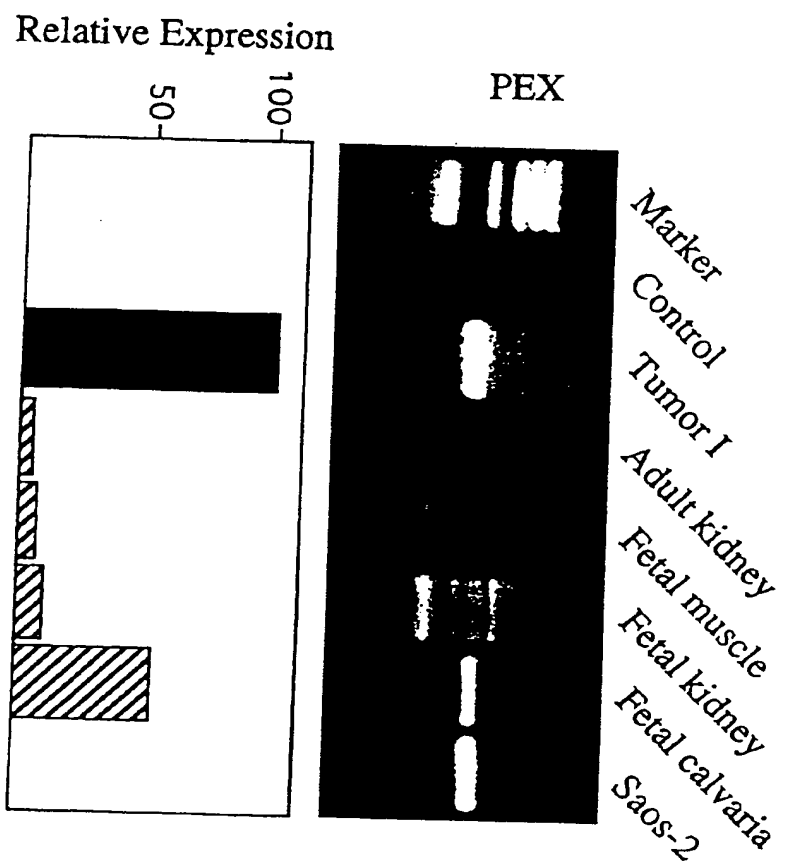


Fig. 3

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Fig. 4

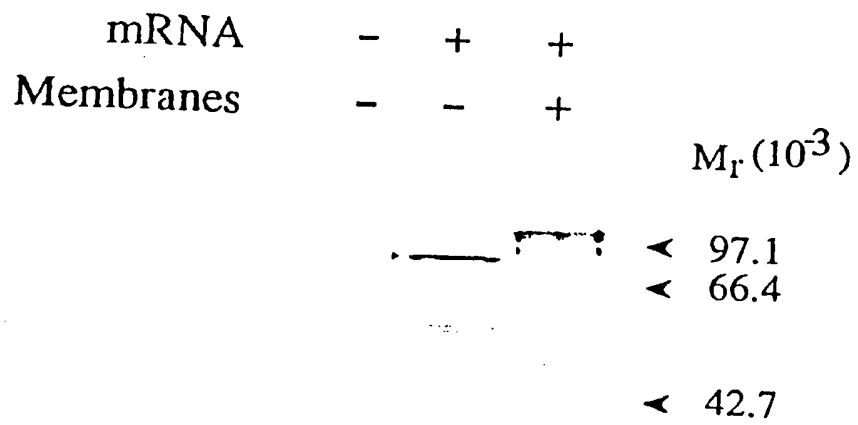
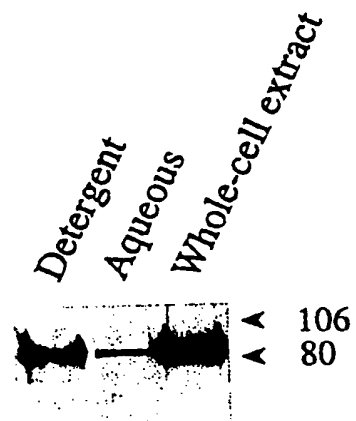


Fig. 5

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B



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Fig. 6

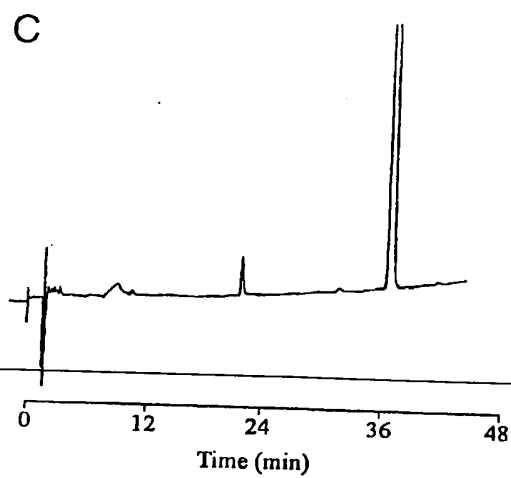
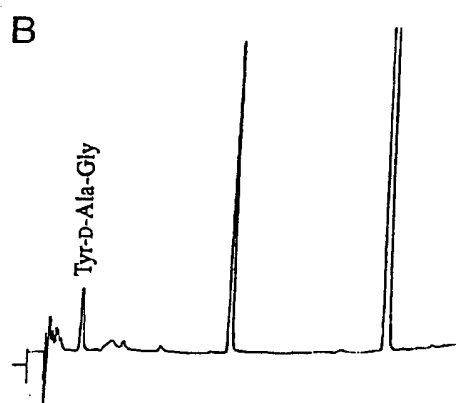
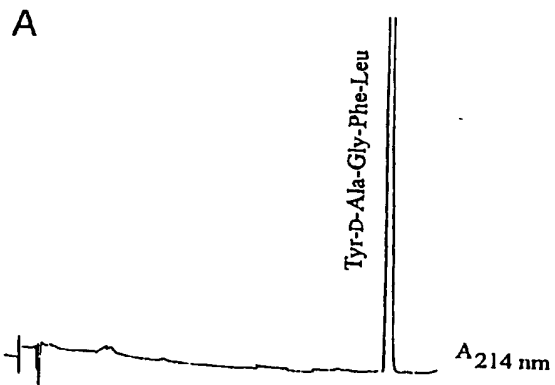


Fig. 7

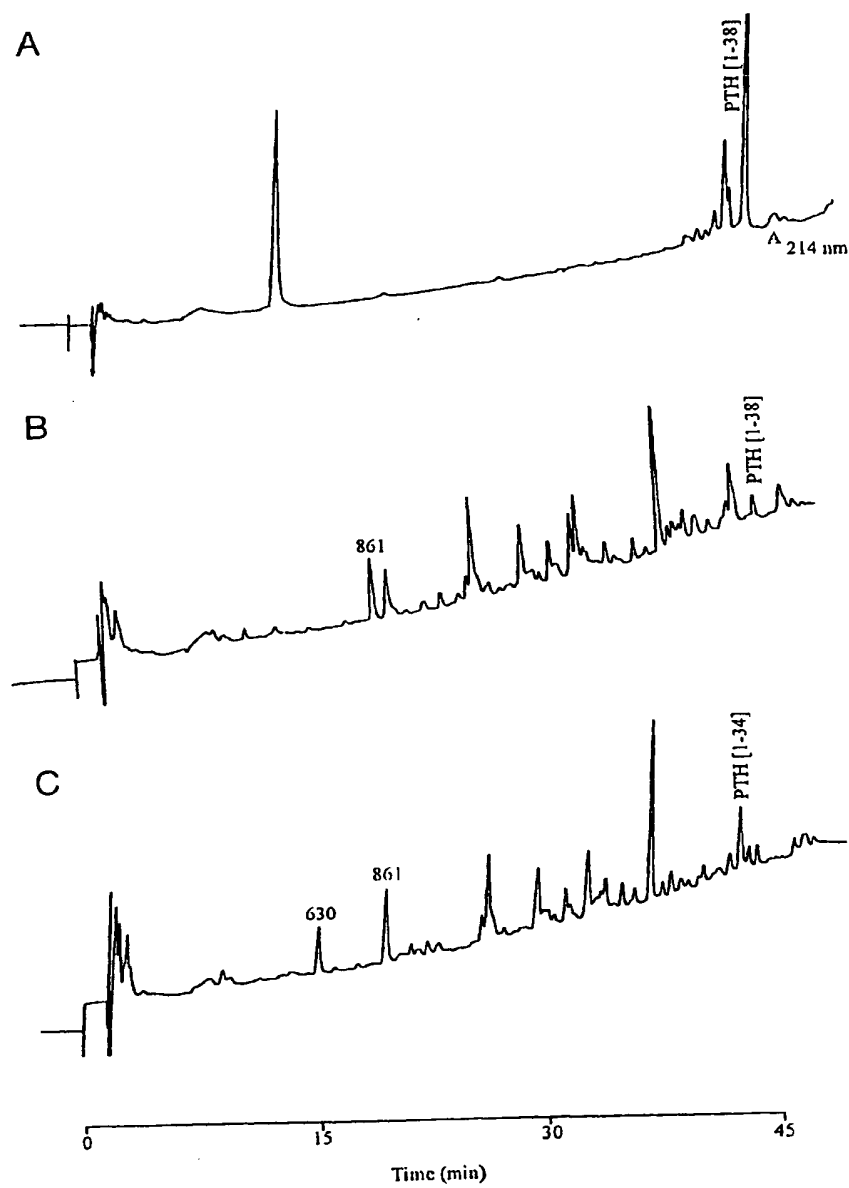


Fig. 8

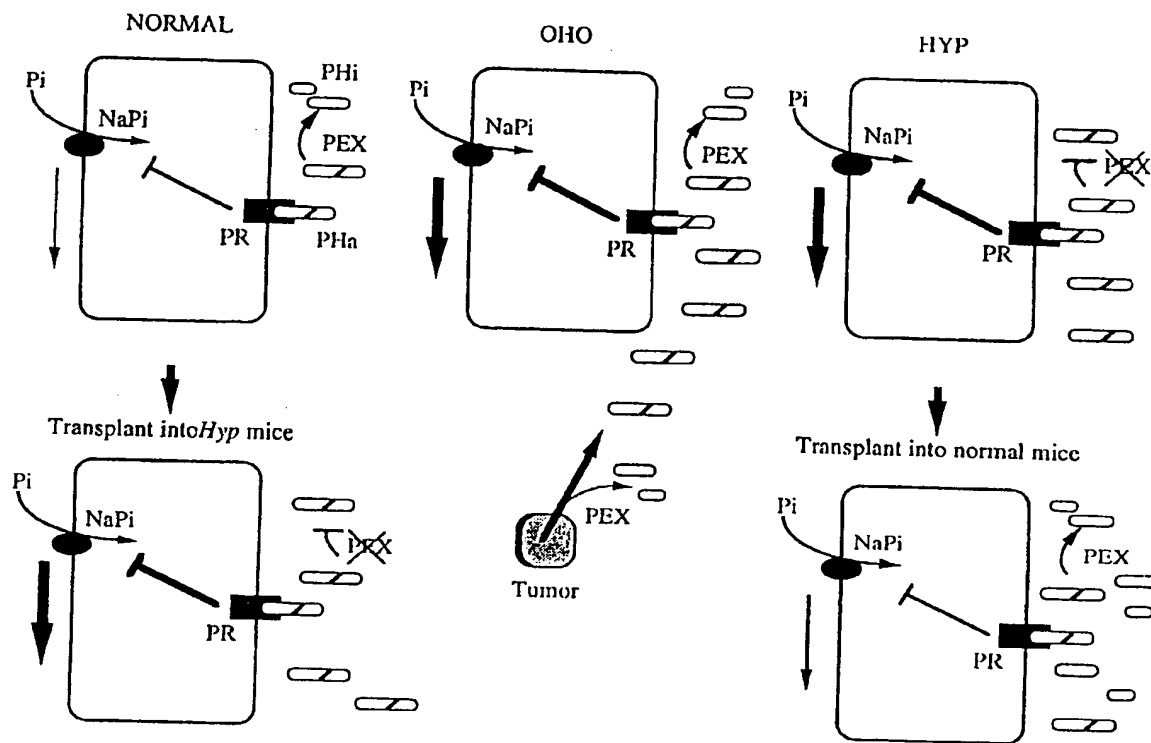


Fig. 9

